Microsatellite Markers for Nuphar japonica (Nymphaeaceae), An Aquatic Plant in the Agricultural Ecosystem of Japan

AUTHOR(S):

Kondo, Toshiaki; Watanabe, Sonoko; Shiga, Takashi; Isagi, Yuji

CITATION:

Kondo, Toshiaki ...[et al]. Microsatellite Markers for Nuphar japonica (Nymphaeaceae), An Aquatic Plant in the Agricultural Ecosystem of Japan. Applications in Plant Sciences 2016, 4(12): 1600082.

ISSUE DATE:

2016-12-08

URL:

http://hdl.handle.net/2433/218923

Microsatellite Markers for *Nuphar japonica* (Nymphaeaceae), An Aquatic Plant in the Agricultural Ecosystem of Japan

Author(s): Toshiaki Kondo, Sonoko Watanabe, Takashi Shiga, and Yuji Isagi
Source: Applications in Plant Sciences, 4(12)
Published By: Botanical Society of America
DOI: http://dx.doi.org/10.3732/apps.1600082
URL: http://www.bioone.org/doi/full/10.3732/apps.1600082

Applications in Plant Sciences

Applications in Plant Sciences 2016 4(12): 1600082

Primer Note

Microsatellite markers for *Nuphar japonica* (Nymphaeaceae), an aquatic plant in the agricultural ecosystem of Japan[1]

Toshiaki Kondo[2,5], Sonoko Watanabe[2], Takashi Shiga[3], and Yuji Isagi[4]

[2]Graduate School for International Development and Cooperation, Hiroshima University, Higashi-Hiroshima 739-8529, Japan; [3]Faculty of Education, Niigata University, Niigata 950-2181, Japan; and [4]Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

- *Premise of the study: Nuphar* species (Nymphaeaceae) are representative aquatic plants in irrigation ponds in Japanese agricultural ecosystems. We developed 15 polymorphic microsatellite markers for *N. japonica* and confirmed their utility for its close relatives *N. oguraensis* var. *akiensis* and *N.* ×*saijoensis*, which originated from natural hybridization between *N. japonica* and *N. oguraensis*.
- *Methods and Results:* Genetic variation was characterized in 15 polymorphic loci in three populations of *N. japonica*. The average number of alleles per locus was 3.47 (range = 2–9; n = 32), and the average expected heterozygosity per locus was 0.84 (range = 0.5–1.0); 11 loci were amplified in *N. oguraensis* var. *akiensis* and 15 in *N.* ×*saijoensis*.
- *Conclusions:* The polymorphic microsatellite markers developed in this study will be useful for investigating the levels of genetic diversity within remnant populations of *Nuphar* taxa and could provide a valuable tool for conservation genetics of these taxa.

Key words: aquatic plant species; cross-amplification; microsatellite; *Nuphar japonica*; Nymphaeaceae.

Species in the genus *Nuphar* Sm. (Nymphaeaceae) are freshwater aquatic plants distributed in the temperate zones of the Northern Hemisphere. The genus is one of the most problematic aquatic macrophytes to identify; seven to 20 species have been recognized worldwide (Cook, 1996; Padgett, 2007), and six species with one or two varieties or forms are distributed in Japan (Shiga et al., 2006; Shiga, 2007; Shiga and Kadono, 2015). In the Saijo Basin (Hiroshima Prefecture, western Japan), three taxa of *Nuphar* are distributed sympatrically (*Nuphar japonica* DC., *N. oguraensis* Miki var. *akiensis* Shimoda, and *N.* ×*saijoensis* (Shimoda) Padgett & Shimoda; Padgett et al., 2002), according to the classification of species based on genetic and morphological variations (Shiga, 2007). *Nuphar japonica* is widely distributed throughout Japan. By contrast, *N. oguraensis* var. *akiensis* grows in a few remote regions of western Japan (this variety also appears in Korea and Taiwan), and *N.* ×*saijoensis*, which originated from natural hybridization between *N. japonica* and *N. oguraensis* (Padgett et al., 2002), grows in several regions of western Japan (Shiga, 2007). Both *N. oguraensis* and *N. oguraensis* var. *akiensis* are listed as *N. pumila* subsp. *oguraensis* (Miki) Padgett in Padgett (2007).

In recent years, aquatic plants growing in irrigation ponds, such as *Nuphar* species, are disappearing owing to land reclamation, repair work, water quality deterioration, and invasion of exotic species. Consequently, *N. oguraensis* is currently included in the Japanese Red Data Book (Ministry of the Environment Japan, 2015). Thus, understanding the genetic diversity of *Nuphar* species will play a key role in its future management. Although microsatellite markers have been developed in two relatives of *N. japonica* (*N. lutea* [Ouborg et al., 2000] and *N. submersa* [Yokogawa et al., 2012]), their utility for *N. japonica* and *N. oguraensis* has been shown to be limited (Yokogawa et al., 2012). Therefore, we have developed polymorphic genomic microsatellite markers for use in genetic investigations of the three *Nuphar* taxa of the Saijo Basin.

METHODS AND RESULTS

We collected plant samples from three populations of *N. japonica* (Sawahara, Kouno, and Doinouchisako-shita ponds), one population of *N. oguraensis* var. *akiensis* (Rakan Pond), and one population of *N.* ×*saijoensis* (Imori-shita Pond) in the Saijo Basin, Hiroshima Prefecture, Japan (Appendix 1); each population was from a separate pond. The geographic distance between individual ponds ranged from 2.3 to 8.4 km, with an average of 5.8 km. We selected Saijo Basin as the study site because it is unique in having three sympatrically distributed *Nuphar* taxa. Sample size was eight or 12 plants per population (48 plants in total). We allowed at least 10 m between sampled individuals to avoid duplicated sampling from the same genet. Pond size and the clonal nature of the species led to small sample sizes for each population. Total genomic DNA was isolated from 30–50 mg of leaf tissue from each plant by using the DNA Suisui–VS extraction buffer (RIZO, Tsukuba, Ibaraki, Japan).

DNA extracted from one *N. japonica* plant collected in Kouno Pond was used for library preparation with a TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, California, USA). Sequencing was performed on a MiSeq

[1]Manuscript received 6 July 2016; revision accepted 20 September 2016.

The authors thank the Program for Leading Graduate Schools (Hiroshima University TAOYAKA program), funded by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT), and a Grant-in-Aid for Scientific Research (no. 16K12640) from MEXT for financial support.

[5]Author for correspondence: kondo@hiroshima-u.ac.jp

doi:10.3732/apps.1600082

Benchtop Sequencer (Illumina) in the 2 × 300-bp read mode, yielding 9,135,890 reads. Prior to assembly, reads were trimmed of adapters and poor quality bases using Trimmomatic version 0.32 (Bolger et al., 2014). Then, reads were assembled into 2,380,573 contigs with fastq-join (Aronesty, 2011). MSATCOMMANDER version 1.0.8 software (Faircloth, 2008) identified 26,667 contigs with dinucleotide motifs with a minimum of 15 repeats. Primers were then designed using Primer3 version 2.2.3 software (Rozen and Skaletsky, 1999) with default settings.

We tested 72 primer pairs. PCR mixtures (10 µL) contained 5 ng of DNA, 0.2 µL of KOD FX Neo polymerase (Toyobo, Osaka, Japan), 5 µL of 2× PCR buffer, 0.4 mM dNTPs, and 0.2 µM of each primer (forward primer with a barcoded split tag [BStag], reverse primer, and fluorescent BStag primer). Each BStag comprised a basal region common among six BStags, a three-nucleotide "barcode" sequence, and a mismatched nucleotide in the middle position. A BStag was added at the 5′ end of the forward primer for each locus to enable postlabeling (Shimizu and Yano, 2011). We labeled the BStag primers with fluorescent dyes to create F9GAC-FAM (5′-CTAGTATCAGGACGAC-3′), F9GTC-VIC (5′-CTAGTATGAGGACGTC-3′), F9TAC-NED (5′-CTAGTATCAGGACTAC-3′), F9GCC-PET (5′-CTAGTATTAGGACGCC-3′), F9CCG-FAM (5′-CTAGTATTAGGACCCG-3′), and F9AGG-VIC (5′-CTAGTATTAGGACAGG-3′) (Shimizu and Yano, 2011). Fragments were amplified in a Veriti Thermal Cycler (Life Technologies, Carlsbad, California, USA) as follows: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and extension at 68°C for 30 s; 12 cycles of 98°C for 10 s, 49°C for 30 s, and 68°C for 10 s; and a final extension at 68°C for 7 min. The size of the PCR products was measured using an ABI PRISM 3130xl Genetic Analyzer and GeneMapper software (both from Life Technologies). We conducted a PCR amplification trial twice for each of four randomly selected individuals from each of the three *Nuphar* taxa, and 15 of 72 primer pairs showed a clear, strong, single band for each allele in *N. japonica.* (The sequences of these 15 primer pairs are listed in Table 1.) Fifteen loci were amplified in *N. japonica* and in *N. ×saijoensis*, and 11 loci were amplified in *N. oguraensis* var. *akiensis* (Table 2).

Evaluation of genetic polymorphism within all 32 *N. japonica* plants showed that the 15 loci had moderate levels of polymorphism. The total number of alleles per locus in these populations ranged from two to nine (mean ± SE: 3.47 ± 0.48). Expected heterozygosity per locus was generally high, ranging from 0.50 to 0.78 (0.56 ± 0.02). At the population level, the number of alleles ranged from two to six (2.47 ± 0.13), observed heterozygosity from 0.50 to 1.00 (0.84 ± 0.02), and expected heterozygosity from 0.45 to 0.74 (0.54 ± 0.01). The levels of polymorphism were moderate in *N. japonica* and *N. ×saijoensis* and relatively low in *N. oguraensis* var. *akiensis* (Table 2). Deviations from Hardy–Weinberg equilibrium in each population and linkage disequilibrium were tested using GENEPOP software, Web version 4.2 (Raymond and Rousset, 1995). Among all loci in all five populations, 11 locus–population combinations deviated significantly from Hardy–Weinberg equilibrium ($P < 0.01$; Table 2). Significant linkage disequilibrium ($P < 0.05$) was observed among six locus pairs in *N. japonica* populations (NJ03362 and NJ14763, NJ04340 and NJ14763, NJ04340 and NJ15714, NJ04340 and NJ18322, NJ14763 and NJ15714, and NJ14763 and NJ18010). Such lack of equilibrium and significant linkage disequilibrium could be explained by the small number of samples in each population and clonal reproduction by rhizome growth in *Nuphar* taxa.

CONCLUSIONS

In this study, a total of 15 polymorphic microsatellite markers for *N. japonica* were developed. Eleven of them (five polymorphic) also amplified in *N. oguraensis* var. *akiensis* and 15 (14 polymorphic) amplified in *N. ×saijoensis*. These markers will be useful both for investigating gene flow among the three taxa of *Nuphar* in the Saijo Basin and for determining the effects of habitat networks on levels of genetic diversity

Table 1. Characteristics of the 15 polymorphic microsatellite markers developed for *Nuphar japonica*.[a]

Locus	Primer sequences (5′–3′)[b]	Repeat motif	Fluorescent label	Allele size range (bp)	GenBank accession no.
NJ03362	F: [F9AGG] AAGGGTAGATGGTGCCGTC R: CGACCCTTGGAGTACGTCAA	$(AG)_{17}$	HEX	384–430	LC164697
NJ03807	F: [F9GAC] CACAGTACCAAACGGCGAAC R: GTAAAGGAAAGGCGACAGGC	$(AG)_{15}$	FAM	127–143	LC164698
NJ03886	F: [F9CCG] CGCGGATTAATGATGGCCTC R: CACCACACCCGTACCTATGT	$(AT)_{17}$	FAM	321–339	LC164699
NJ04340	F: [F9CCG] GAAACCCACACATCACCTCC R: GGACGTAGCATTCTCTCTCCT	$(AG)_{16}$	FAM	312–328	LC164700
NJ08140	F: [F9GAC] ATCTCTCCCGCATCAAGACC R: CTCGATCTCCACCTTCAGCA	$(AG)_{18}$	FAM	136–170	LC164701
NJ08349	F: [F9GCC] AGCATAACCACACAAAGTCCC R: CCAATGAGCTCACCTTTCCC	$(AG)_{18}$	PET	274–288	LC164702
NJ11253	F: [F9CCG] AGGACACATTTGCCAACGTG R: CCATTATCGCCGGCAAGAAG	$(AG)_{17}$	FAM	316–328	LC164703
NJ12418	F: [F9GTC] TTTGTTGGTTGGCACGAGAC R: CGAAGGTAGGGCTGTGAACT	$(AG)_{18}$	HEX	186–196	LC164704
NJ13401	F: [F9GCC] ACACAAATTCACGGAGCAGAC R: CAGCTTGGGTCTTGAATGGAC	$(AC)_{17}$	PET	289–315	LC164705
NJ14763	F: [F9AGG] GGCAAGACAAAGTGAGGCTC R: GGCTGGTTTGGGTTCAGTTC	$(AT)_{15}$	HEX	366–370	LC164706
NJ15714	F: [F9GCC] GCATTCCTGATCGTGTCTGC R: CAACCCTACAGAAGAGCGGA	$(AG)_{16}$	PET	250–270	LC164707
NJ17517	F: [F9GAC] GAGCAAGGAGGAGAAGGTTTC R: CTCTATAATGGCGACACAAGCT	$(AG)_{17}$	FAM	146–174	LC164708
NJ18010	F: [F9TAC] CATCACGACGGAACCAAAGG R: GCACGAGCGAGACTAGAAGA	$(AG)_{18}$	NED	228–260	LC164709
NJ18322	F: [F9AGG] TTCACAGCTCCTCTTCCGTC R: GCTCGAGAACCTTGACCTCA	$(AG)_{16}$	HEX	406–410	LC164710
NJ21829	F: [F9AGG] ACGCACACCAATCGTTGTAG R: CTACCCAGAAGCGACAGTGA	$(AG)_{17}$	HEX	401–433	LC164711

[a]Annealing temperature was 60°C for all loci.

[b]Sequences of the BStag primers: F9GAC-FAM = 5′-CTAGTATCAGGACGAC-3′, F9GTC-VIC = 5′-CTAGTATGAGGACGTC-3′, F9TAC-NED = 5′-CTAGTATCAGGACTAC-3′, F9GCC-PET = 5′-CTAGTATTAGGACGCC-3′), F9CCG-FAM = 5′-CTAGTATTAGGACCCG-3′, F9AGG-VIC = 5′-CTAGTATTAGGACAGG-3′.

TABLE 2. Genetic variation of the 15 polymorphic microsatellite loci in three populations of *Nuphar japonica*, one population of *N. oguraensis* var. *akiensis*, and one population of *N.* ×*saijoensis*.[a]

		N. japonica								*N. oguraensis* var. *akiensis*			*N.* ×*saijoensis*		
		Sawahara (n = 12)			Kouno (n = 12)			Doinouchisako-shita (n = 8)			Rakan (n = 8)			Imori-shita (n = 8)	
Locus	A_T	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
NJ03362	9	6	1.000	0.736	3	0.917*	0.554	2	0.750	0.500	—	—	—	6	0.375*	0.858
NJ03807	2	2	0.917	0.518	2	0.666	0.464	2	1.000	0.533	1	0.000	0.000	2	0.750	0.533
NJ03886	3	2	0.833	0.507	3	0.833	0.562	2	0.875	0.525	—	—	—	2	0.125	0.125
NJ04340	3	2	0.833	0.507	3	0.833	0.554	2	0.875	0.525	2	1.000*	0.500	2	0.875	0.525
NJ08140	3	3	0.750	0.620	3	0.583	0.453	2	0.875	0.525	2	0.000	0.233	3	0.625	0.492
NJ08349	3	3	0.833	0.562	2	0.833	0.507	2	1.000	0.533	2	0.000	0.400	2	0.125	0.125
NJ11253	4	2	1.000*	0.522	3	0.666*	0.638	3	0.875*	0.642	—	—	—	1	0.000	0.000
NJ12418	3	3	0.917	0.562	2	0.833	0.507	2	0.875	0.525	2	0.125	0.125	2	0.750	0.500
NJ13401	2	2	0.666	0.464	2	1.000*	0.522	2	1.000	0.533	1	0.000	0.000	2	0.875	0.525
NJ14763	2	2	0.583	0.518	2	0.750	0.489	2	1.000	0.533	1	0.000	0.000	3	0.625	0.542
NJ15714	4	2	0.833	0.521	2	0.666	0.464	4	1.000*	0.650	2	0.000	0.233	2	0.750	0.500
NJ17517	3	2	1.000*	0.522	2	0.750	0.489	2	0.875	0.525	1	0.000	0.000	3	1.000	0.592
NJ18010	6	3	0.500	0.507	3	0.750	0.562	5	1.000*	0.700	1	0.000	0.000	3	0.750	0.700
NJ18322	2	2	0.833	0.522	2	0.583	0.489	2	0.875	0.525	1	0.000	0.000	2	0.875	0.525
NJ21829	3	2	1.000*	0.522	2	0.833	0.507	3	0.875	0.692	—	—	—	2	0.000	0.233

Note: — = not amplified; A = total number of alleles in *N. japonica* populations; H_e = expected heterozygosity; H_o = observed heterozygosity.
[a]Voucher and locality information are provided in Appendix 1.
* Significant deviation from Hardy–Weinberg equilibrium expectations ($P < 0.01$).

within the populations of these taxa. The results may have important implications for aquatic plant conservation and restoration.

LITERATURE CITED

ARONESTY, E. 2011. ea-utils: Command-line tools for processing biological sequencing data. Website https://expressionanalysis.github.io/ea-utils/ [accessed 26 October 2016].

BOLGER, A., M. LOHSE, AND B. USADEL. 2014. TRIMMOMATIC: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120.

COOK, C. D. K. 1996. Aquatic plant book. SPB Academic Publishing, Amsterdam, The Netherlands.

FAIRCLOTH, B. C. 2008. MSATCOMMANDER: Detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* 8: 92–94.

MINISTRY OF THE ENVIRONMENT JAPAN. 2015. Red Data Book 2014–Threatened Wildlife of Japan, Vol. 8: Vascular Plants. Gyousei, Tokyo, Japan.

OUBORG, N. J., W. P. GOODALL-COPESTAKE, P. SAUMITOU-LAPRADE, I. BONNIN, AND J. T. EPPLEN. 2000. Novel polymorphic microsatellite loci isolated from the yellow waterlily, *Nuphar lutea. Molecular Ecology* 9: 497–498.

PADGETT, D. J. 2007. A monograph of *Nuphar* (Nymphaeaceae). *Rhodora* 109: 1–95.

PADGETT, D. J., M. SHIMODA, L. A. HORKY, AND D. H. LES. 2002. Natural hybridization and the imperiled *Nuphar* of western Japan. *Aquatic Botany* 72: 161–174.

RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249.

ROZEN, S., AND H. SKALETSKY. 1999. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SHIGA, T. 2007. A systematic study of *Nuphar* (Nymphaeaceae) in Japan with special reference to the role of hybridization. Ph.D. dissertation, Kobe University, Kobe, Japan.

SHIGA, T., J. ISHII, Y. ISAGI, AND Y. KADONO. 2006. *Nuphar submersa* (Nymphaeaceae), a new species from central Japan. *Acta Phytotaxonomica et Geobotanica* 57: 113–122.

SHIGA, T., AND Y. KADONO. 2015. *Nuphar saikokuensis* (Nymphaeaceae), a new species from central to western Japan. *Journal of Japanese Botany* 90: 20–26.

SHIMIZU, T., AND K. YANO. 2011. A post-labeling method for multiplexed and multicolored genotyping analysis of SSR, indel and SNP markers in single tube with bar-coded split tag (BStag). *BMC Research Notes* 4: 161.

YOKOGAWA, M., T. SHIGA, S. KANEKO, AND Y. ISAGI. 2012. Development of nuclear microsatellite markers for the critically endangered freshwater macrophyte, *Nuphar submersa* (Nymphaeaceae), and cross-species amplification in six additional *Nuphar* taxa. *Conservation Genetics Resources* 4: 295–298.

Appendix 1. Voucher information for populations of *Nuphar* species used in this study. One voucher was collected from each population sampled.

Species	Pond name[a]	Geographic coordinates	Voucher collection no.[b]
N. japonica DC.	Sawahara	34°24′11″N, 132°44′13″E	Watanabe0001
N. japonica	Kouno	34°25′24″N, 132°41′24″E	Watanabe0002
N. japonica	Doinouchisako-shita	34°21′42″N, 132°44′04″E	Watanabe0003
N. oguraensis Miki var. *akiensis* Shimoda	Rakan	34°26′11″N, 132°44′20″E	Watanabe0004
N. ×*saijoensis* (Shimoda) Padgett & Shimoda	Imori-shita	34°21′54″N, 132°42′32″E	Watanabe0005

[a] Populations are located in the Saijo Basin, Hiroshima Prefecture, Japan.
[b] All vouchers were deposited in the Herbarium of the Graduate School for International Development and Cooperation, Hiroshima University, Hiroshima, Japan.